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Higher doses of bisphosphonates further improve bone mass, architecture, and strength but not the tissue material properties in aged rats

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ABSTRACT

We report the results of a series of experiments designed to determine the effects of ibandronate (Ibn) and risedronate (Ris) on a number of bone quality parameters in aged osteopenic rats to explain how bone material and bone mass may be affected by the dose of bisphosphonates (BP) and contribute to their anti-fracture efficacy.

Eighteen-month old female rats underwent either ovariectomy or sham surgery. The ovariectomized (OVX) groups were left untreated for 2 months to develop osteopenia. Treatments started at 20 months of age as follows: sham and OVX control (treated with saline), OVX + risedronate 30 and 90 (30 or 90 μg/kg/dose), and OVX + ibandronate 30 and 90 (30 or 90 μg/kg/dose). The treatments were given monthly for 4 months by subcutaneous injection. At sacrifice at 24 months of age the 4th lumbar vertebra was used for μCT scans (bone mass, architecture, and degree of mineralization of bone, DMB) and histomorphometry, and the 6th lumbar vertebra, tibia, and femur were collected for biomechanical testing to determine bone structural and material strength, cortical fracture toughness, and tissue elastic modulus. The compression testing of the vertebral bodies (LVB6) was simulated using finite-element analysis (FEA) to also estimate the bone structural stiffness.

Both Ibn and Ris dose-dependently increased bone mass and improved vertebral bone microarchitecture and mechanical properties compared to OVX control. Estimates of vertebral maximum stress from FEA were correlated with vertebral maximum load ($r=0.5$, $p<0.001$) and maximum stress ($r=0.4$, $p<0.005$) measured experimentally. Tibial bone bending modulus and cortical strength increased compared to OVX with both BP but no dose-dependent effect was observed. DMB and elastic modulus of trabecular bone were improved with Ibn 30 compared to OVX but were not affected in other BP-treated groups. DMB of tibial cortical bone showed no change with BP treatments. The fracture toughness examined in midshaft femurs did not change with BP even with the higher doses. In summary, the anti-fracture efficacy of BP is largely due to their preservation of bone mass and while the higher doses further improve the bone structural properties do not improve the localized bone material characteristics such as tissue strength, elastic modulus, and cortical toughness.

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Introduction

Bisphosphonates (BP) are the primary drugs used to treat osteoclast-induced bone loss due to osteoporosis, Paget disease, malignancies metastatic to bone, multiple myeloma, and hypercalcemia of malignancy [1,2]. The bone anti-resorptive activity of BP has been associated with preservation of trabecular microarchitecture, improvement in bone strength in animal models and with reduction in new fractures in clinical studies [3–5]. Recent reports on subtrochanteric insufficiency fractures in patients treated for 2–5 years with alendronate have attributed the fractures to excessive suppression of bone remodeling [6,7], although no biochemical or histological data were presented. The excessive suppression of bone resorption brought about by BP therapy may result in reduced bone material properties with microdamage accumulation, excess degree of mineralization of bone (DMB), changes in mineral-matrix properties, crystal size, and bone stiffness, all of which

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may compromise the structural performance of bone [4,8,9]. Currently, there is an interest in clinical practice to treat osteoporosis with high doses of BP given intravenously or orally to allow for less frequent administration and improved patient compliance [10–12]. The data on the effects of higher doses of BP in lowering bone turnover rate especially of cortical bone and the consequent bone material is limited [8,9] warranting further investigations on influences of excessive suppressed bone remodeling, either with high doses of BP or long-term treatment regimen, on bone mechanical integrity.

Studies that have evaluated the change in bone quality with BP treatment began after a number of clinical studies found a rapid and significant reduction in risk of vertebral fractures after BP treatment with modest increases in areal BMD [13,14]. Although the volumetric bone density, which is not traditionally measured in clinical studies, is highly correlated with bone strength and strains [15–18], recent studies have suggested that changes in bone quality, in addition to bone quantity, may also exert effects on fracture risk reduction following anti-resorptive agents [19,20]. Several studies in animal models have reported that BP might increase, in a dose-dependent manner, microdamage accumulation even though no apparent adverse consequences on whole bone mechanical properties have been reported [8,9] nor has the clinical impact of microdamage potentially induced by BP been established in humans. In addition, an increasing number of cases of osteonecrosis of the jaw in patients treated with BP have been reported in the literature, raising concern about quality changes in bone with BP, although the disease has a wide range of incidence depending on the population under study and other co-factors that are not fully established [21].

To further elucidate the dose–response of BP on bone strength and material properties, we compared the effects of ibandronate (Ibn) and risedronate (Ris) in aged ovariectomized (OVX) rats treated with doses comparable to, or higher than, the clinical doses prescribed for the treatment and prevention of postmenopausal osteoporosis and used by Delmas et al. [22,23], Black et al. [10], and Lewiecki et al. [11]. Similar doses based on a subcutaneous route of administration in OVX rats [24] or IV injection in OVX monkeys [25] have been reported to inhibit bone loss and improve vertebral bone strength. We studied bone mass, architecture, and biomechanical improvements with respect to bone material changes with higher doses of BP.

Materials and methods

Animals and experimental procedures

A total of 84 eighteen-month old female Fischer 344 rats were purchased from NIA (Bethesda, MD) and maintained on commercial rodent chow (Rodent Diet; Teklad, Madison, WI) with 0.95% calcium and 0.67% phosphorus. The rats were kept in a room with 21 °C and a 12-h light/dark cycle. The study protocol was approved by UC Davis Institutional Animal Care and Use Committee. Rats were randomized by body weight into six groups ($n = 14$/group) and underwent ovariectomy except for one group that underwent sham surgery. The ovariectomized (OVX) groups were left untreated for 2 months to develop osteopenia. The experimental groups started their treatments at 20 months of age (day 0 experiment) and included sham and OVX control (treated with saline), risedronate 30 (Ris 30, 30 μg/kg/dose), risedronate 90 (Ris 90, 90 μg/kg/dose), ibandronate 30 (Ibn 30, 30 μg/kg/dose), and ibandronate 90 (Ibn 90, 90 μg/kg/dose). The treatments were given on days 0, 30, 60, and 90 of the experiment by subcutaneous injection and rats were killed at 24 months of age. All animals received calcein (10 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) subcutaneously at 14 and 4 days before sacrifice for bone histomorphometric measurements of surface-based bone turnover. The 4th lumbar vertebral body (LVB4) was dissected and placed in 10% phosphate-buffered formalin for 48 h prior to examinations by micro-computed tomography (μCT) scans and histomorphometry. The LVB6 and tibia were collected for biomechanical testing and femurs were collected for fracture toughness measurements. Further, the compression test of the vertebral bodies was simulated using FEA to estimate bone material stiffness and the apparent modulus [26]. The data collected from μCT were also used to calculate DMB.

Micro-CT measurements of bone architecture and degree of mineralization of bone

Three-dimensional scans and evaluation of the LVB4 and tibial cortex from each rat was obtained using μCT (*viva* CT 40, Scanco Medical, Bassersdorf, Switzerland) at energy level of 70 kVp and intensity of 85 μA with an isotropic resolution of 10.5 μm in all three spatial dimensions. In the LVB4, a 2.3 mm-thick cross section from the center of the vertebral body was scanned for evaluation of the trabecular and cortical bone (Fig. 1). Tibial cortical bone was scanned in the region starting 8 mm proximal to the tibial–fibular junction for 0.6 mm towards the midshaft. The grayscale images were segmented using a constrained 3D Gaussian filter (sigma = 1.0, support = 1.0) and a threshold of 284 for vertebral trabecular, 264 for vertebral cortical, and 400 for tibial cortical bone to evaluate the trabecular and cortical bone volume (BV/TV) for each sample. In addition, vertebral trabecular connectivity density, number (Tb N), and thickness (Tb Th) were evaluated for each experimental group. The X-ray coefficient of attenuation (cm^{-1}) of each voxel was used to obtain the histogram of bone mineral concentration and mean DMB within the region of the vertebrae scanned for architectural analyses. The material attenuation coefficient from grayscale intensities is proportional to calcium concentration of bone [27,28]. The mineral concentration values were standardized with a manufacturer supplied phantom of five different hydroxy apatite densities embedded in soft-tissue equivalent resin. The mineralization profile was studied after excluding the partial volume by peeling two voxels from trabecular bone surface and described by mean DMB [20,27–29].

Bone histomorphometry measurements

The LVB4 was dehydrated in ethanol and embedded undecalcified in methylmethacrylate for sectioning with a Leica RM 2265 microtome (Leica Microsystems Nussloch GmbH, Germany) into 4- and 8-μm thick sections. The 4-μm sections were stained with tetrachrome to measure bone volume, architecture, and osteoclast surface (Oc.S/BS) with the light microscope, whereas the 8-μm sections were left unstained for measurement of surface-based histomorphometric indices using a fluorescent microscope (Nikon Eclipse E400, Japan) linked to an image analysis software (Bioquant Image Analysis Corporation, Nashville, TN). Bone turnover measurements included single- and double-labeled perimeter, interlabel width, and osteoclast surface. The parameters were used to calculate the mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) according to the standard guidelines previously published [30].

Biomechanical testing

The mechanical properties of bone yield and maximum stress, fracture toughness, and tissue elastic modulus were determined using lumbar compression, tibial and femoral three-point bending tests, and nano-indentation modulus mapping. The LVB6 was tested using an axial compression test after removal of the vertebral end plates using a wafer saw. Prior to loading the relevant cross-sectional dimensions and the height of the specimen was measured using an optical microscope with a 0.5 μm resolution (Olympus STM-UM Measuring Microscope, Olympus American Inc., Melville, NY). The sample was

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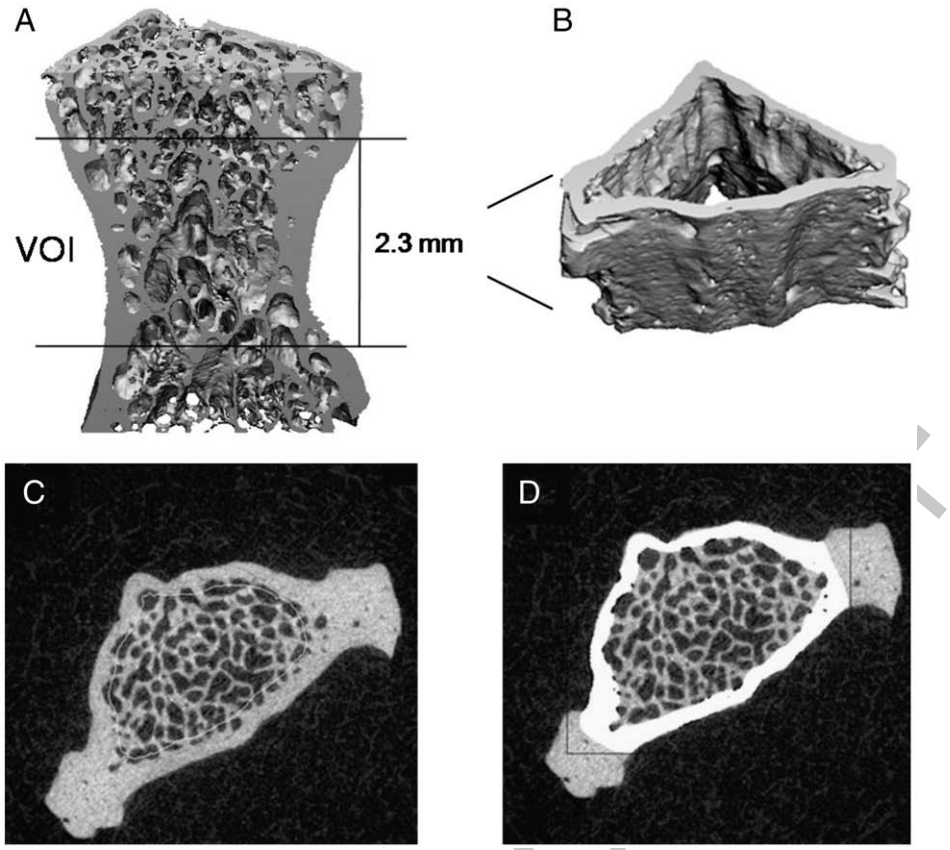

Fig. 1. High resolution *ex vivo* μCT images of vertebral body (LVB4) in an intact 2 years old rat; (A) 3D reconstruction of the vertebral body showing longitudinal cut-away view and the volume of interest (VOI) selected for architectural, mineralization, and finite element analyses ($n = 14$/group) from 1/3 of the total height in the center using a voxel size of 10.5 μm; (B) 3D image of a volume of cortical bone from the same region analyzed after excluding the trabecular bone; (C) and (D) 2D images showing the region of interest for analyses of trabecular bone and cortical shell, respectively.

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loaded to failure at a displacement rate of 0.01 mm/s. A similar displacement rate was applied for measuring the three-point bending strength of the tibiae using an electroservo-hydraulic materials testing system (MTS Model 831, Eden Prairie, MN). Left femurs were used for fracture toughness measurements determined in notched three-point bending tests. The ends of bones were cut off with a low speed saw, then notched and loaded such that the posterior surface was in tension and the anterior surface in compression. Notches were cut into the cortical midshaft region on the posterior surface of the bone using a low speed saw to about 1/3 of the diameter by one observer and were sharpened by polishing in diamond paste with a standard razor blade to a root radius of ~5–10 μm. The samples were tested in three-point bending with the maximum load and crack length recorded to assess the toughness. Details have been recently published by this group [31]. The broken halves of bones were then dehydrated and the fracture surface was examined in an environmental SEM (Hitachi S-4300SE/N ESEM, Hitachi America). The femoral and tibial cross-sectional area and second moment of inertia were calculated numerically from the SEM image taken using ImageJ software from fracture surfaces. Stresses and strains were computed in accordance with the methods described by Akhter et al. [32]. The yield stress was determined as the stress at 0.2% plastic strain and the maximum stress as the stress at peak load. Bending and compression modulus values were calculated from the slope of the linear region of the stress–strain curve. Fracture toughness values were defined at the maximum stress, using the procedures described in [31] for the toughness evaluation of small animal bone.

In addition, microstructural bone matrix material properties were determined at the trabecular region of the proximal tibiae ($n = 5$/group) using a nano-indenter (Triboscope, Hysitron, Inc.) integrated with an atomic force microscope (AFM, Dimension 3000, Digital Instruments). The tibial metaphyseal bone samples were embedded undecalcified in methylmethacrylate for modulus mapping. The technique is well established [33,34] and has been used to create modulus maps of human dentin in which differences in dentin elastic and viscoelastic micromechanical response was quantified [35,36]. The nano-indenter was operated in nano-DMA mode in which the indenter interacted with the bone surface in direct force modulation, similar to AFM force modulation with the exception that the modulated force is known with much more accuracy using the indenter than with the AFM cantilever. At each pixel location, the storage modulus (indicating the elastic properties of the bone tissue material) of the matrix material was determined with pixel resolution determined by the shape of the indenter tip.

Finite element modeling of the vertebrae

In order to determine the individual contributions of the cortical shell and the trabecular core to the overall vertebral body biomechanical performance, the segmented μCT grayscale image of the vertebral body from each animal ($n = 14$/group) was incorporated into a finite element model to analyze its biomechanical behavior [37]. The original image voxels were directly converted to elements and based on the grayscale intensity values obtained from μCT each element segmented as bone was assigned a Young's modulus of 18 GPa and a Poisson's ratio of 0.3 [38]. The boundary conditions that defined the load platen/specimen interface were assumed to be frictionless. The Young's modulus in compression was calculated for the whole vertebrae, the cortical shell, and the isolated trabecular

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Table 1
Mean values and standard error of vertebral (LVB4) histomorphometric parameters of 2 year old rats treated for 4 months with ibandronate (Ibn) or risedronate (Ris) at 30 or 90 μg/kg monthly doses.

	Sham	OVX + saline	OVX + Ibn 30	OVX + Ibn 90	OVX + Ris 30	OVX + Ris 90
Bone volume/total volume (%)	32 ± 1[a]	23 ± 3	36 ± 1[a]	39 ± 2[a]	36 ± 2[a]	39 ± 2[a]
OC surface/bone surface (%)	2.6 ± 0.3[a]	6.5 ± 0.4	2.9 ± 0.2[a]	2.1 ± 0.2[a,b]	3.2 ± 0.6[a]	2.6 ± 0.3[a]
Mineralizing surface/bone surface (%)	5.7 ± 0.8[a]	8.1 ± 1.5	2.7 ± 0.4[a]	2.9 ± 0.5[a]	2.5 ± 0.6[a]	2.1 ± 0.3[a]
Mineral apposition rate (μm/day)	1.51 ± 0.06[c]	1.65 ± 0.12	1.21 ± 0.11[a]	1.10 ± 0.14[a]	1.13 ± 0.06[a]	1.27 ± 0.20[a]
Bone formation rate/bone surface ($\mu m^3/\mu m^2$/day)	8.1 ± 1.1[c]	12.0 ± 3.2	4.2 ± 1.0[a]	4.1 ± 0.9[a]	3.3 ± 1.0[a]	3.1 ± 11.3[a]

[a] Comparison to OVX is significant at $p \leq 0.001$.
[b] Comparison to lower dose of the drug is significant at $p \leq 0.05$.
[c] Comparison to OVX is significant at $p \leq 0.05$.

Bone Details of the numerical method has been published elsewhere [39,40].

Statistical analysis

The group means and standard errors of the means (SE) were calculated for all outcome variables. ANOVA (release 15; Minitab) was used to compare the groups for bone parameters. An effect was considered significant if $p \leq 0.05$. For parameters with significant differences determined by ANOVA, inter-group differences were determined with the Tukey pairwise post-hoc test with the Bonferroni correction for multiple comparisons. Correlations were performed using the Pearson test. The nano-indentation test results were analyzed using a non-parametric test of Kruskal–Wallis.

Results

The body weight of the rats was not affected by drug administration; however, as compared to the sham group, ovariectomy resulted in significant increases in body weight within 2 weeks after the surgery, which was maintained throughout the experiment despite pair feeding (data not shown).

Histomorphometry

Histomorphometric results are presented in Table 1. The BV/TV was significantly lower in L4 vertebrae of the OVX group vs. sham and was restored to the sham levels in the Ris 30 and Ibn 30 groups, and was significantly higher than sham in Ris 90 and Ibn 90 groups ($p<0.01$). The OVX group also showed a significantly higher osteoclast surface to bone surface (Oc. surface/BS) than the sham group ($p<0.001$), which was recovered to sham-control levels in all drug groups. A significant increase in dynamic parameters of MS/BS, MAR, and BFR was also observed as a result of ovariectomy, indicating higher bone turnover, which were all suppressed with both Ris and Ibn treatments to the levels significantly lower than untreated OVX and sham animals. A dose–response effect was not generally observed in histomorphometric parameters, except that oscteoclast suppression was higher with Ibn at higher dose as measured by Oc. surface/BS ($p<0.05$) (Table 1).

Micro-CT of trabecular and cortical bone mass, architecture, and degree of mineralization of bone

Table 2 shows the changes in bone mass and architecture in response to BP. The OVX group without treatment showed significant changes in trabecular structural parameters (Table 2) indicating the development of OVX-induced bone loss. Significant recoveries from the OVX level were observed for structural parameters studied in Ibn and Ris groups. The Ibn and Ris treatments were similar for all of the structural parameters when given at the same dose. However, with each BP treatment there was a dose–response effect for trabecular BV/TV, thickness, and BS/BV ($p \leq 0.05$). Similarly, the BV/TV loss of cortical bone in the OVX group recovered to sham levels with Ibn and Ris treatments but also increased with the higher dose of each drug.

Compared to the sham group, ovariectomy significantly decreased the mean DMB in both trabecular (1091 vs. 1071 mg/cm^3) and cortical bone (1195 vs. 1170 mg/cm^3) of the vertebrae and the cortical bone of the tibia (1321 vs. 1289 mg/cm^3). Treatment with both Ibn and Ris corrected the loss of mineral in trabecular bone tissue. In the vertebral cortical bone BP treatment did not affect bone

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Table 2
Indices of trabecular and cortical bone microarchitecture and mineralization by micro-CT in 2 year old ovariectomized rats treated for 4 months with ibandronate (Ibn) or risedronate (Ris) at 30 or 90 μg/kg monthly doses (mean ± SE).

	Sham	OVX + saline	OVX + Ibn 30	OVX + Ibn 90	OVX + Ris 30	OVX + Ris 90
Vertebral trabecular bone						
Bone volume/total volume (%)	37 ± 1[a]	26 ± 1	39 ± 2[a]	47 ± 3[a,b]	40 ± 4[a]	48 ± 5[a,b]
Connectivity density (1/mm^3)	42.1 ± 3.8[a]	21.8 ± 1.9	31.4 ± 1.6[a]	32.0 ± 2.0[a]	33.8 ± 2.8[a]	31.5 ± 2.9[a]
Trabecular number (1/mm)	3.0 ± 0.1[a]	2.2 ± 0.08	3.9 ± 0.1[a]	4.3 ± 0.2[a]	4.1 ± 0.2[a]	4.6 ± 0.3[a]
Trabecular thickness (mm)	0.086 ± 0.002[a]	0.078 ± 0.004	0.104 ± 0.003[a]	0.124 ± 0.009[a,b]	0.109 ± 0.008[a]	0.129 ± 0.013[a,b]
Trabecular separation (mm)	0.32 ± 0.01[a]	0.49 ± 0.02	0.24 ± 0.01[a]	0.21 ± 0.01[a]	0.23 ± 0.01[a]	0.20 ± 0.02[a]
Bone surface/bone volume (1/mm)	27.2 ± 1.0[a]	32.5 ± 1.4	20.7 ± 0.7[a]	17.8 ± 1.1[a,b]	20.1 ± 1.5[a]	17.2 ± 1.8[a,b]
Mean degree of mineralization of bone (mgHA/cm^3)	1091 ± 5[a]	1071 ± 10	1096 ± 4[a]	1080 ± 3	1082 ± 7	1080 ± 6
Vertebral cortical bone						
Bone volume/total volume (%)	89 ± 1[a]	86 ± 1	90 ± 1[a]	92 ± 1[a,b]	89 ± 1[a]	91 ± 1[a,b]
Mean degree of mineralization of bone (mgHA/cm^3)	1195 ± 6[a]	1170 ± 3	1180 ± 4	1165 ± 6[b]	1177 ± 6	1150 ± 10[a,b]
Tibial cortical bone						
Bone volume/total volume (%)	76 ± 0.6[a]	73 ± 1	77 ± 0.5[a]	79 ± 0.9[a,b]	77 ± 1[a]	80 ± 1[a,b]
Mean degree of mineralization of bone (mgHA/cm^3)	1321 ± 6[a]	1289 ± 3	1294 ± 4	1293 ± 4	1280 ± 5	1286 ± 6

[a] Comparison to OVX is significant at $p \leq 0.001$.
[b] Comparison to lower dose of the drug is significant at $p \leq 0.05$.

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Table 3
Mechanical properties of vertebra, tibia, and femur in 2 year old ovariectomized rats treated for 4 months with ibandronate (Ibn) or risedronate (Ris) at 30 or 90 μg/kg monthly doses (mean ± SE).

	Sham	OVX + saline	OVX + Ibn 30	OVX + Ibn 90	OVX + Ris 30	OVX + Ris 90
Vertebrae compression						
Maximum load (N)	223.7 ± 20.0[a]	173.6 ± 23.5	240.0 ± 20.6[a]	291.7 ± 18.2[a,b]	241.9 ± 30.7[a]	285.9 ± 30.4[a,b]
Yield stress (MPa)	29.7 ± 4.1[a]	19.3 ± 4.7	31.5 ± 3.0[a]	36.8 ± 4.4[a]	34.3 ± 4.7[a]	33.8 ± 6.6[a]
Maximum stress (MPa)	33.8 ± 2.6	27.5 ± 1.1	39.6 ± 2.3[a]	41.2 ± 4.0[a]	38.4 ± 4.6[a]	44.8 ± 5.2[a]
Modulus (GPa)	0.49 ± 0.18[a]	0.39 ± 0.11	0.69 ± 0.12[a]	0.70 ± 0.80[a]	0.61 ± 0.12[a]	0.59 ± 0.86[a]
Tibiae bending						
Maximum load (N)	56.4 ± 2.2	64.8 ± 2.5	61.1 ± 1.0	59.8 ± 1.8	62.3 ± 3.2	63.2 ± 2.4
Maximum stress (MPa)	142.0 ± 7.4[a]	99.0 ± 13.2	122.3 ± 7.4[a]	113.7 ± 6.1[a]	109.5 ± 7.4[a]	114.9 ± 6.7[a]
Modulus (GPa)	5.5 ± 0.5[a]	2.6 ± 0.5	4.0 ± 0.3[a]	4.0 ± 0.3[a]	3.8 ± 0.4[a]	3.9 ± 0.3[a]
Nano-indentation (proximal tibia metaphyses)						
Elastic modulus (GPa)	12.0 ± 1.0[a]	9.8 ± 0.5	12.7 ± 0.8[a]	10.5 ± 0.4[b]	10.6 ± 0.5	10.3 ± 0.7
Femoral toughness (MPa √m)	3.7 ± 0.2	3.5 ± 0.2	4.0 ± 0.2	3.5 ± 0.2	3.7 ± 0.2	3.1 ± 0.2
Vertebral compression estimates by FEA						
Stiffness (N/mm)	23743 ± 916[a]	20970 ± 1939	27580 ± 1700[a]	35247 ± 1866[a,b]	27135 ± 1706[a]	36054 ± 2801[a,b]
Apparent modulus (GPa)	5.29 ± 0.29	5.09 ± 0.37	6.43 ± 0.21[a]	7.24 ± 0.35[a,b]	6.48 ± 0.53[a]	7.39 ± 0.57[a,b]
Average trab. tissue stress (MPa)	119.7 ± 1.6	120.2 ± 0.8	130.7 ± 1	131.8 ± 2.1	129.4 ± 2.9	131.1 ± 2.9
Load carried by Tb. bone (%)	24.8 ± 2.2	25.3 ± 4.2	32.0 ± 2.2[a]	27.4 ± 2.0	31.1 ± 2.4[a]	38.8 ± 3.9[a]
Load carried by Ct. bone (%)	75.2 ± 2.2	74.4 ± 4.3	68.0 ± 2.2[a]	72.6 ± 2.0	68.9 ± 2.4[a]	61.2 ± 3.9[a]

[a] Comparison to OVX is significant at $p \leq 0.001$.
[b] Comparison to lower dose of the drug is significant at $p \leq 0.05$.

mineralization however higher dose compared to the lower dose appeared to have a ~1.7% decrease ($p<0.05$) on mean DMB as assessed from coefficients of X-ray attenuation obtained by μCT (Table 2). DMB of tibial midshaft cortex did not change with BP treatment as compared to the OVX or with the higher doses.

Mechanical testing for whole and localized bone strength

Mechanical test results as shown in Table 3 were obtained from all experimental groups for lumbar compression, tibia three-point bending tests, femur fracture toughness tests, and tibial trabecular nano-indentation test. Based on the vertebral compression tests, there was nearly a 40% improvement in both maximum load and maximum stress in the OVX-treated groups. However, the response was dose dependent with both BP treatments only for maximum load and not for the maximum stress. Maximum loads in vertebral compression were positively correlated ($p<0.001$) with BV/TV of the trabecular bone ($r=0.72$), trabecular thickness ($r=0.73$), and trabecular number ($r=0.65$), and negatively correlated ($r=-0.34$, $p<0.01$) with DMB, whether sham and OVX groups were included or not (Fig. 2). Tibial mechanical testing in OVX group showed lower values compared to sham only for bending modulus and maximum stress but not for maximum load. Ibn and Ris treatment increased tibial bending modulus and maximum stress ($p<0.05$) but the tibial maximum load was not affected by BP treatments. There was no dose-dependent effect of BP on mechanical behavior of tibia, a site that is more than 90% composed of cortical Bone The fracture toughness measured by notched three-point bending of femurs increased non-significantly compared to OVX but showed a decreasing trend with higher doses of each BP, albeit not significant. Results from nano-indentation test of trabecular bone at proximal tibia indicated that tissue elastic modulus was increased compared to OVX only with Ibn at the lower dose and was similar to OVX in both Ris groups and with higher dose of Ibn treatment.

Discussion

The changes in bone mass and quality with high doses of BP or long-term treatments are currently under investigation [41,42]. Treatment with ibandronate and risedronate of 2 year old OVX rats restored vertebral bone mass dose-dependently and had no adverse effect on trabecular mineralization, elastic modulus, cortical fracture toughness, or the estimated material properties such as bone maximum stress, a parameter that is independent of bone size and shape [43]. Lumbar compressive strength was increased as a result of improvements of bone mass and architecture by BP treatments and was more correlated with the trabecular than the cortical bone volume.

The efficacy of various continuous and intermittent regimens of BP has been investigated in experimental models of osteoporosis. Intravenous monthly bolus injections of Ibn at 30 or 150 μg/kg for 16 months in OVX monkeys suppressed bone turnover and improved spinal and femoral neck bone mass and strength dose-dependently [25]. As for the clinical data, the approved oral regimens of weekly and monthly Ris and monthly Ibn have not been evaluated in fracture-endpoint trials. Once-monthly oral Ibn (150 mg) for 12 months was shown in postmenopausal osteoporotic women to improve volumetric BMD and bone strength estimated by FEA of QCT scans [11]. Also, the twice a month 75 mg or once a-month 150 mg Ris regimens were shown as effective as 5 mg daily doses in improving lumbar spine BMD and reducing biochemical markers of bone turnover (uNTX and sBAP) after 1 year treatment [22,23].

Since bone mass and maximum load increased with the dose of BP, as shown in this study and by others [44], it is reasonable to hypothesize that high doses of BP, or long-term treatments, may change the material properties and exert deleterious effects on bone mechanics as has been reported in recent clinical observations of site-specific insufficiency in bone strength [6,7,45]. The fractures have been reported to possibly develop from over suppression of bone turnover by prolonged alendronate therapy. Although very recent data from analyses of cross sectional and alendronate cohort studies indicate that subtrochanteric-diaphyseal femur fractures share the epidemiology and characteristic nature of osteoporotic fractures [46]. The excessive suppression of bone remodeling by high BP is also thought to compromise bone integrity by accumulation of microdamage [8]. However, the microdamage accumulation has been suggested to peak during the early period of BP treatment and does not continue to accumulate with longer treatment periods [9]. Also, even though a relationship between microdamage accumulation and an altered toughness at the material level has been found [8], the other determinants of bone strength including ultimate load, stiffness, and energy to failure or other material properties estimates including bone maximum stress and modulus were reported unaffected after 3 years of daily alendronate treatment in a preclinical animal model [9]. Other

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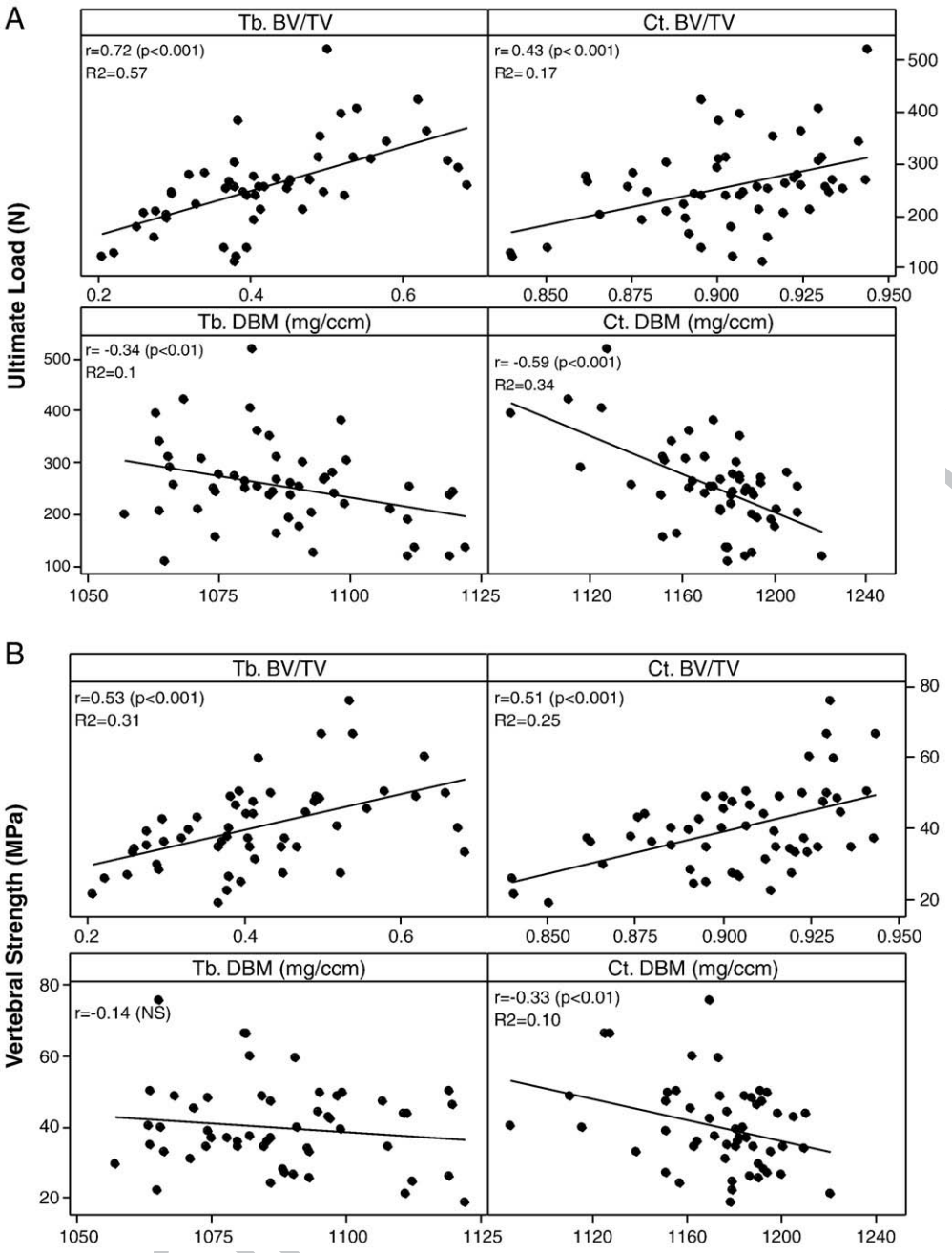

Fig. 2. Fitted line plots of vertebral strength in 2 years old rats vs. bone mass and degree of mineralization of bone at (A) structural and (B) material levels, as corrected for bone geometry. Tb. and Ct. BV/TV, trabecular and cortical bone volume/total volume. DBM, degree of mineralization of Bone *r*, Pearson correlation, and R^2, coefficient of determination obtained from linear regression model with a single predictor variable. NS, not significant.

material properties of bone may be affected including bone tissue mineralization and the quality of collagen and mineral crystal [47,48], which are affected by bone turnover [49] and are potently suppressed with BP treatment. The link between DMB and mechanical resistance has been reported [50–52]. Higher bone mineralization has been found to be beneficial in increasing bone strength or reducing incidence of new fractures [14,28]; however, excessive mineralization may increase bone stiffness and, in some cases, brittleness [53], suggesting tissue degree of mineralization can be biomechanically important.

We studied bone mineralization and tissue elastic modulus, cortical fracture toughness, and yield and maximum stress for material properties. Mean DMB of cortical bone in the vertebrae appeared to decrease with the higher doses of both BP. Given the very low turnover rate in cortical bone, this may likely be due to a further suppression of bone remodeling, and therefore bone mineralization, or an alteration of primary mineralization with high doses of BP. The DMB measured with μCT is an apparent DMB and may not precisely reflect the DMB of the bone tissue as measured by quantitative microradiography [54].

Although a negative correlation of maximum load and DMB was found for vertebral compression tests, the reductions in vertebral cortical DMB did not influence the outcome mechanical properties of vertebrae including bone maximum load, yield and maximum stress. The changes in cortical DMB were not perhaps large enough to adversely affect the *ex vivo* mechanical behavior of bone, similar to findings of microdamage accumulation as discussed earlier, or more likely, the increased bone mass and improved microarchitecture of trabecular bone observed with higher doses of BP compensated for

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the possible adverse effects of DMB changes, if any. Compared to the rapid architectural and bone mass changes following BP therapy [20], DMB changes with BP treatment is a very slow and gradual process that may take much longer to affect Bone These effects may compromise the mechanical properties of bone at the tissue level, independent from the structure, as seen in human subtrochanteric insufficiency fractures [6]. Interestingly, our results of trabecular nano-indentation test were in agreement with the trabecular DMB data obtained from micro-CT in that the higher dose of Ibn reduced both the DMB and tissue elastic modulus, compared to the lower dose, and set these parameters back to the levels observed in OVX group. However, this did not interfere with the dose dependent increase in vertebral maximum load or did not negatively affect the estimated material properties obtained from the mechanical tests. Except in disease or with anti-resorptive treatments, the mineral density distribution of cancellous human bone has been reported to be essentially constant with age, ethnicity, skeletal site, and gender [55]. Boivin et al. [54] reported that after 2 years of alendronate therapy in postmenopausal osteoporotic women, the trabecular DMB was 7.5% higher than placebo as measured by microradiography on transiliac bone biopsies. The increase in DMB was nearly 11% after 3 years of alendronate, although this was not statistically different than after 2 years of treatment. Nevertheless, such increases in DMB is reflected in BMD [51] which is associated with a reduced incidence of fractures [56]. Even with anabolic therapies such as PTH (1–34), which induces a biphasic DMB and significantly lowers the mean DMB, there is a rapid improvements in bone mechanical properties in pre- and clinical studies [42,57], and a large increase in BMD, compared to BP, that appears to offset the adverse effects of low DMB on mechanical behavior [58].

Femoral fracture toughness was not affected by BP although appeared to have a tendency to decrease with higher doses of Ibn and Ris. The coefficient of variation for fracture toughness has been reported to be over 30% [59] and therefore variations in the fracture toughness of the bone samples must be at least 20% to draw meaningful conclusions [31]. Fracture toughness is an estimation of the material properties of the cortical bone which affect the whole bone fracture resistance and is especially relevant if crack-like defects may be present, e.g., with higher doses of BP or prolonged treatment periods [9].

The effects of the BP treatment on vertebral bone stiffness estimated from micro-CT data and FEA were similar to those of bone mass and architectural parameters. The basic assumptions in FEA are that the structure is fully connected and there are fixed and homogeneous material properties. Two-component FEA includes trabecular and cortical bone voxels and assigns a constant 18 GPa modulus to each element segmented as mineralized tissue using the threshold value. Estimates for the elastic modulus of the individual trabeculae of human cancellous bone are reported to vary from 1 to 20 GPa [60] depending on orientation of specimen and axial stress condition. We found that trabecular, but not the cortical, maximum stress estimated from FEA, were correlated to experimental maximum load ($r=0.5$) and stress ($r=0.4$) ($p<0.001$). The FEA suggested that the percent load carried by trabecular bone increased in BP-treated groups, likely due to cancellous bone preservation by BP, and no dose–response effect was noticed. The significant correlation of FEA data with *ex vivo* mechanical tests provides support that this observation is likely valid. These data may explain why BP treatment, by reducing trabecular bone turnover, improve trabecular bone strength and reduce fractures in anatomic areas such as the lumbar spine that have a high percentage of trabecular Bone

Our study has a number of important features, specifically the dose–response assessment of several different measurements of bone quality in aged animals with two BP used for osteoporosis treatment. Nevertheless, there are some limitations to our study. First, our experimental period was only 4 months and although clinical studies find BP treatment can improve strength and reduce incident vertebral fractures, longer treatments may be required to determine BP effects on cortical bone quality. In clinical practice, patients have been treated for over 10 years with BP, and short-term animal studies will not provide sufficient information on the chronic effects of these medications on localized bone quality and strength [6,7,61,62]. Second, we did not observe a change in localized fracture toughness in our study. Since the fracture toughness measurement is associated with a higher variability than other tests of bone strength, we would have needed a larger sample size to make it easier to control for this limitation. Also, we used only two doses of BP; inclusion of higher doses in the study might have provided additional information on the effects on cortical bone quality. Finally, we did not perform finite element modeling on the vertebral samples used for compression testing to assess the cortical bone contribution to the compression bone strength affected by BP treatment. However, we performed FEA analysis from the lumbar vertebrae data obtained from the micro-CT scans. We found that BP treatment increased the load carried by the trabecular bone and this is consistent with the clinical observation that BP improve trabecular bone mass and architecture and prevent incident vertebral compression fractures [63,64].

In summary, we found that the improved bone mass and trabecular architecture involved with higher doses of BP treatments were markedly associated with improved whole bone mechanical behavior as measured by maximum load and FEA parameters. However, higher doses of BP did not further increase the estimated material properties, or bone fracture toughness, or tissue elastic modulus. These results suggest that the possible bone material changes with BP may not be reflected in the structural improvements and that the trabecular and cortical bone compartments have different responses to these medications. Additional studies on BP that assess the differences in whole bone and localized material properties over a longer experimental time period and the clinical correlations with regard to the nature of the fractures (osteoporotic or atypical) are clearly needed.

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